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Differentiation-inducing activity of lupane triterpenes on a mouse melanoma cell line

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Abstract Lupane triterpenes were found to promote melanogenesis, a hallmark of B16 2F2 mouse melanoma cell differentiation. Studies of the structure-activity relationships demonstrated that the keto function at C-3 of the lupane skeleton played important roles in the melanogenic activities of lupane triterpenes on melanoma cells. The carbonyl group at C-17 of lupane triterpenes was essential against their apoptosis-inducing activity against human cancer cells via the inhibition of topoisomerase I. We investigated whether signaling mechanisms were involved in the stimulative effects of lupane triterpenes on the melanogenesis of B16 2F2 cells. In experiments using selective inhibitors against various signal transduction molecules and Western blotting analysis, it was suggested that p38 MAPK was involved in melanoma cell differentiation as a downstream effector of PKA. Lupeol (compound **1**), a lupane triterpene, induced dendrite formations, a morphological hallmark of B16 2F2 cell differentiation by rearrangement of the actin cytoskeleton. The activation of cofilin, an actin depolymerizing factor, by compound **1** caused actin fiber disassembly in B16 2F2 cells. Furthermore, compound **1** was shown to inhibit the cell motilities of human melanoma and neuroblastoma in vitro.

Keywords Lupane triterpene · Melanoma · Differentiation · Dendritic formation · Signaling mechanism

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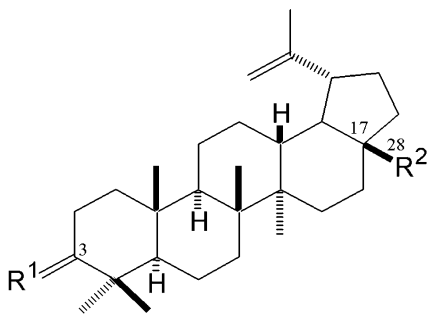
Introduction

The mouse melanoma cell line B16 was isolated from C57 BL/6 mice and is known to produce melanin pigments (Filder 1975). Some studies demonstrated that B16 cells can be induced to differentiate into mature melanocyte-like cells by treatment with cAMP-elevating agents such as α-melanocyte-stimulating hormone (α-MSH, Engalo et al. 1995), signal transduction pathway inhibitors (Busca et al. 1996; Engalo et al. 1998), and mannosylerythritol lipid (Zhao et al. 1999). In the differentiation of B16 cells, up-regulation of melanin biosynthesis and dendrite outgrowths is observed; however, information concerning melanoma differentiation inducers is very limited. In the present study, we isolated lupane triterpenes as differentiation inducers of mouse melanoma cells from Compositae plants, and investigated the structure-activity relationships and signaling mechanisms involved in melanoma cell differentiation.

Differentiation-inducing compounds on melanoma cells

Isolation of melanogens from Compositae plants

Briefly, we selected B16 mouse melanoma-derived sub-clone (B16 2F2) with high differentiation capability. This clone is a non-melanogenic sub-clone under normal cell culture conditions; however, the melanin synthesis of B16 2F2 cells was up-regulated by melanogens such as α-MSH, and the accumulation of melanin pigments was able to be detected under light microscopic observation. Based on these findings, we screened the melanogenic activities in food materials on B16 2F2 cells without the determination of melanin contents. The activity of B16 2F2 cells was detected widely in Compositae plants, brown algae and some mushrooms such as *Tricholoma* sp. (Hata et al. 2000). By bioassay-guided fractionation, we isolated 4 lupane triterpenes (compounds **1**–**4**, Fig. 1) as melanogens from two Compositae plants, Chinese dandelion root and *Lactuca indica* (Hata et al. 2000; Hata et al. 2003a). The cell growth and melanin contents after treatment with compound **1** (= lupeol) at various concentrations for 72 h are shown in Fig. 2. Melanin biosynthesis was induced by the addition of compound **1** at more than 5 μM; and proliferation of the melanoma cells was inhibited at 20 μM (Fig. 2). Even at 50 μM, the cells did not detach and remained > 95% viable. These results suggested that the effect of compound **1** on B16 2 F2 cells might not be cytotoxic but cytostatic. Lupane is one of the major triterpenes in Compositae plants and its biological activities have been reported (Akihisa et al. 1996; Saleem et al. 2004); however, there have been no reports concerning the differentiation-inducing activity of lupane triterpenes on tumor cells. Therefore, we studied the actions of lupane triterpenes on melanoma cells in more detail.



Compound	R^1	R^2
1	α-H, β-OH	CH_3
2	=O	CH_3
3	α-H, β-OAc	CH_3
4	α-H, β-$OCOC_{15}H_{31}$	CH_3
5	α-H, β-OH	CH_2OH
6	=O	CH_2OH
7	α-H, β-OAc	CH_2OCOCH_3
8	α-H, β-OH	CHO
9	=O	CHO
10	α-H, β-OH	COOH
11	α-H, β-OH	$COOCH_3$
12	=O	$COOCH_3$

Fig. 1 Chemical structures of lupane triterpenes

Structure-activity relationships of melanogenesis and apoptosis induction

To investigate the relationships between the structures of these triterpenes and their melanogenic activity, B16 2F2 cells were treated with 12 lupane triterpenes for 72 h, and the intracellular melanin content and the growth of B16 2F2 cells were measured (Hata et al. 2002). The results are summarized in Table 1. The differences at C-17 had little influence on their melanogenic activity; however, oxidation of the OH group at C-3 markedly decreased their ED_{50} values (compounds **2**, **6**, **9** and compound **12**), and the long fatty acid group at C-3 decreased activity (compound **4**). The structural differences at C-3 play an important role in the

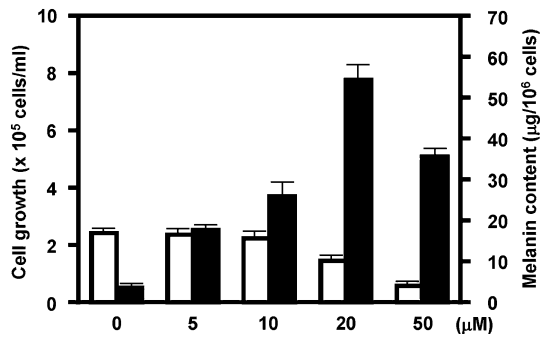


Fig. 2 Melanogenic effects of compound **1** against B16 2F2 cells. B16 2F2 cells (1×10^5 cells) were treated with various concentrations of compound **1** for 72 h, the cell growth (□) and melanin content (■) were measured

Table 1 Effects of lupane triterpenes on B16 2F2 cell melanogenesis

Compound	ED_{50} (μM)[a]	IC_{50} (μM)[b]
1	9.9	38.0
2	0.35	25.4
3	7.5	22.7
4	24.7	26.9
5	4.8	27.4
6	0.19	36.8
7	3.3	29.3
8	3.4	6.4
9	0.35	4.1
10	4.1	7.9
11	2.5	4.9
12	0.13	5.6

[a] ED_{50} value was determined from the result of dose-response experiments in which the up-regulation of melanogenesis by the compound was monitored

[b] IC_{50} value represents the concentrations that inhibit cell growth by 50%

induction of B16 2F2 cell melanin synthesis by lupane series triterpenes. Other pentacyclic triterpenes such as oleanane and ursane could not differentiate B16 2F2 cells. Previously, it was reported that compound **10** was a selective anticancer agent against some human cancer cell lines (Fulda et al. 1999; Pisha et al. 1995; Schmidt et al. 1997), and the carboxyl group at the C-17 was essential for apoptotic activities (Lee et al. 1998). In our studies of anti-proliferative effects by these triterpenes on B16 2F2 cells, compounds **8–12**, all of which have a carbonyl group at C-17, were found to inhibit B16 2F2 cell proliferation at lower concentrations than other compounds. Furthermore, the condensation and fragmentation of nuclei was observed in B16 2F2 cells treated with compound **8**, indicating that these compounds showed apoptotic effects on B16 2F2 cells (Fig. 3). In this study, compounds **10–12**, all of which possess a carboxyl group at C-17, showed strong cytotoxic effects against B16 2F2 cells, but compound **8** and compound **9** inhibited B16 2F2 cell growth to the same extent as compounds **10–12**. These results suggested that the carbonyl group and not the carboxyl group at C-17 was essential for the apoptotic effects of lupane triterpenes. In addition, ketone formation at C-3 of lupane triterpenes enhanced their melanogenic activities. We concluded that the different moieties of

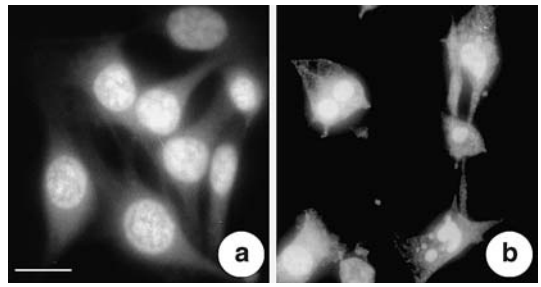


Fig. 3 Induction of apoptosis by compound **8**. B16 2F2 cells were incubated without, (**a**) or with 10 μM compound **8** for 48 h, and stained with Hoechst 33258 dye (bar, 50 μm)

the lupane skeleton separately regulated the induction of differentiation and apoptosis of B16 2F2 melanoma cells.

The carbonyl group at C-17 of lupane triterpenes is essential for their apoptotic activity against B16 2F2 cells. We examined the selective cytotoxity of lupane triterpenes against human cancer cell lines (Hata et al. 2003b). Compounds **8–12** with a carbonyl group at C-17 markedly inhibited human cancer cell growth but not normal fibroblasts (Table 2). In particular, compound **9** selectively inhibited leukemia and lung cancer cell growth by the induction of apoptosis. Additionally, compound **9** inhibited the proliferation of two drug-resistant leukemia cells to the same extent as parent cells. Topoisomerase I (Topo I) inhibitors are known to induce cancer cell apoptosis, and compound **10** was shown to be a potent inhibitor of eukaryotic Topo I (Tabata et al. 2001; Chowdhury et al. 2002). Compounds **8–12** with a carbonyl group at C-17 markedly inhibited human Topo I, but compound **1** and compound **5** did not affect on the enzyme activity (Fig. 4). Our study suggested that lupane triterpenes with a carbonyl group at C-17 should be selective anti-cancer agents by the inhibition of Topo I, and are effective against not only parent cancer cells, but also drug-resistant cells.

Signaling mechanisms involved in melanoma cell differentiation

Melanogenesis

Some studies showed the involvement of cAMP-PKA or PKC signaling in the up-regulation of melanocyte/melanoma cell melanogenesis (Park and Gilchrest 1999; Tachibana 2000; Zhao et al. 2001). More

Table 2 Cytotoxic activity profile of compound **9** against human cancer cells

Cell Line (Tissue)	IC_{50} (μM)[a]
HL60 (leukemia)	0.48
U937 (leukemia)	1.5
K562 (leukemia)	1.8
K562/ADM(adriamycin-resistant K562)	1.7
K562/VCR (vincristine-resistant K562)	2.0
G361 (melanoma)	9.4
SK-MEL-28 (melanoma)	9.3
GOTO (neuroblastoma)	7.8
NB-1 (neuroblastoma)	8.8
A549 (lung)	2.3
LU65 (lung)	2.4
Lu99 (lung)	0.81
MG63 (bone)	17.9
Saos2 (bone)	13.5
SH-10-TC (stomach)	5.8
MKN45 (stomach)	9.6
HCT-15 (colon)	7.2
MIA Paca2 (pancreas)	8.1
ACHN (renal)	9.4
HepG2 (liver)	9.3
MCF-7 (breast cancer)	14.7
HeLa (cervix)	2.1
Ca Ski (cervix)	6.3
OVK18 (ovary)	10.2
EJ-1 (urinary bladder)	16.2
T24 (urinary bladder)	12.5
WI38 (normal fibroblast)	>20

[a] IC_{50} value represents the concentrations that inhibit cell growth by 50%

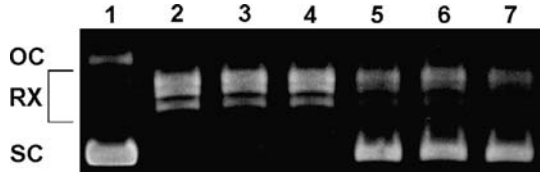


Fig. 4 Topo I inhibitory activity of lupane triterpenes. Superoiled plasmid DNA was incubated with human Topo I in the absence or presence of lupane triterpenes. Supercoiled plasmid DNA alone (lane 1), Topo I-treated supercoiled DNA in the absence (lane 2), or presence of 5 μM compound **1** (lane 3), compound **5** (lane 4) or compounds **8**–**10** (lanes 5–7). The positions of supercoiled (SC), open circle (OC) and relaxed (RX) DNA were indicated

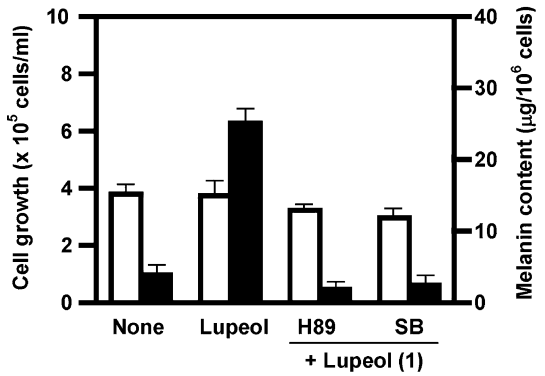


Fig. 5 Effects of H89 and SB203580 on the Melanogenesis of B16 2F2 Cells by compound **1**. Following treatment with B16 2F2 cells alone, or with 5 μM H89 or 5 μM SB203580 (SB) in the absence or presence of 10 μM compound **1** for 72 h, the cell growth (▭) and melanin content (■) were measured

recently, the p38 MAPK cascade was demonstrated to play an important role in α-MSH-induced melanogenesis (Smalley and Eisen 2000). The signaling mechanisms involved in compound **1**-induced melanogenesis were studied using various signal pathway inhibitors and by Western blotting (Hata et al. 2003c). PKA inhibitor (H89) and p38 MAPK inhibitor (SB203580) abolished the activation of melanogenesis induced by compound **1** (Fig. 5). Western blot analysis revealed that compound **1** transiently induced the activation of p38 MAPK in B16 2F2 cells (Fig. 6). Furthermore, the inhibition of PKA blocked the activation of p38 MAPK by compound **1**, indicating that p38 MAPK is a downstream target of the cAMP-PKA pathway. In the studies of structure-activity relationships, the structural differences at C-3 of lupane triterpenes were very important in their melanogenic activity. Following the addition of 10 μM compound **1** or compound **2**, the melanin biosynthesis of B16 2F2 melanoma cells was up-regulated as compared with the untreated controls

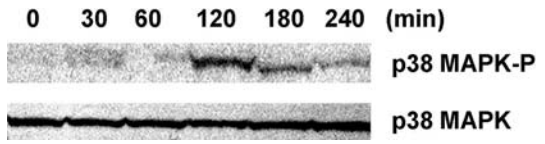


Fig. 6 Activation of p38 MAPK by compound **1**. B16 2F2 cells were incubated with 10 μM compound **1** for 0–240 min (lanes 1–6), and then cell lysates were analyzed by Western blotting using anti-phospho p38 MAPK (p38 MAPKP) antibody (upper panel) or anti-p38 MAPK antibody (lower panel)

(Fig. 7). No up-regulation was observed with 1.0 μM compound **1**, but 1.0 μM compound **2** induced cell melanogenesis to the same extent as stimulation with 10 μM. Phosphorylation of p38 MAPK increased with 10 μM compound **1**, when compared to untreated B16 2F2 cells, but the level of phospho-p38 MAPK in 1.0 μM compound **1**-treated B16 2F2 cells was very low; however, phospho-p38 MAPK was detected in B16 2F2 cells treated with 1.0 and 10 μM compound **2**. These findings showed that the structure differences at C-3 of lupane triterpene are important in the activation of p38 MAPK in B16 2F2 cells, and the structure-activity relationship in the activation of p38 MAPK signaling affects the up-regulation of B16 2F2 cell melanogenesis by lupane triterpenes.

Dendrite formation

Melanosomes are lysosome-related organelles and contain all the components required to synthesize melanin pigments such as tyrosinase, a key enzyme in melanin biosynthesis. In response to hormones and UV irradiation, melanin pigments are synthesized in melanosomes, which are transferred from the tips of melanocyte dendrites to the surrounding keratinocytes to protect against UV damage or carcinogenic effects (Boissy 2003; Okazaki et al. 1976; Seiberg 2001). Studies have demonstrated that the dendritic outgrowth of melanocyte/melanoma cells is promoted by UV irradiation, cAMP-elevating agents and growth factors, as well as by the induction of melanogenesis; therefore, dendrites are recognized as a morphological indicator of melanocyte/melanoma cell differentiation (Hara et al. 1995; Yoshida et al. 2000). We investigated the formation of dendrites in B16 2F2 cells incubated with compound **1**. Untreated B16 2F2 cells were mainly round without dendrites, but the morphology of B16 2F2 cells incubated with compound **1** for 8–12 h was markedly changed, and dendricity was promoted. As the rearrangements of cytoskeletal components result in the dendrite outgrowth of B16 cells (Busca et al. 1998), we studied whether the remodeling of cytoskeletal components such as microfilaments and microtubules, was involved in the formation of dendrites in B16 2F2 cells (Fig. 8). In untreated

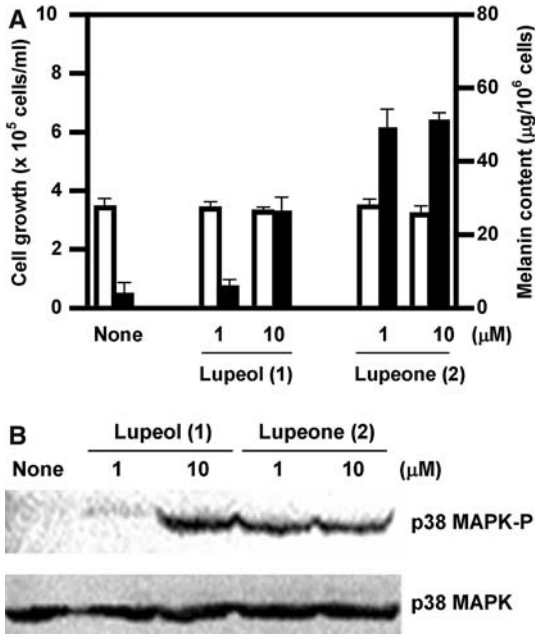


Fig. 7 Structure-activity relationships of lupane triterpenes as to the activation of melanogenesis and p38 MAPK. (**A**): following treatments with B16 2F2 cells with lupane triterpenes for 72 h, cell growth (□) and melanin content (■) were measured. (**B**): B16 2F2 cells were incubated with 1 or 10 μM lupane triterpenes for 0–240 min, and the cell lysates were analyzed by Western blotting with anti-phospho p38 MAPK antibody (upper panel) or anti-p38 MAPK antibody (lower panel)

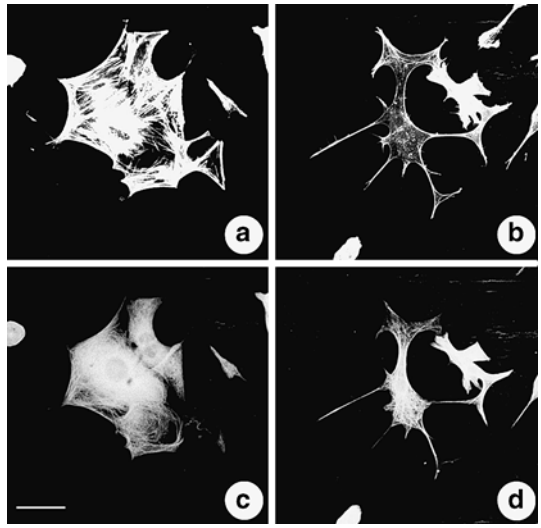


Fig. 8 Effect of compound **1** on cytoskeletal components in B16 2F2 cells. B16 2F2 cells were cultured without (**a** and **c**) or with 10 μM compound **1** (**b** and **d**) for 12 h, stress fibers were stained with Alexa Fluor 488 phalloidin (**a** and **b**), and the microtubular network was detected with anti-α-tubulin antibody using rhodamine-conjugated secondary antibody (**c** and **d**), respectively (bar, 50 μm)

B16 2F2 cells, actin appeared organized in stress fibers crossing the cytoplasm. By treatment with compound **1**, stress fiber assembly in the cytoplasm was disrupted, leaving phalloidin-labeled F-actin only in the dendrites; however, compound **1** did not influence the remodeling of the microtubular network. The expression levels of actin and α-tubulin in B16 2F2 cells treated with compound **1** remained constant. Recently, signaling mechanisms involved in the disassembly of actin stress fibers have been studied, and the inactivation of Rho signaling was shown to be involved in the dendrite outgrowth of B16 cells (Scott 2002). Rho up-regulates the formation of stress fibers through the activation of downstream effectors such as Rock. Rock and Rho-kinase activate LIM-kinases, which are responsible for the phosphorylation and inactivation of actin depolymerization factors such as cofilin (Buchan et al. 2002; Maekawa et al. 1999; Sumi et al. 1999). In contrast to the mechanisms of cofilin inactivation by LIM-kinases, Slingshot was found to dephosphorylate phospho-cofilin and reactivate cofilin to depolymerize F-actin (Niwa et al. 2002). We examined the levels of phospho-cofilin in B16 2F2 cells stimulated with compound **1**. The levels of phospho-cofilin (inactive form) in B16 2F2 cells decreased by treatment with compound **1** in a time-dependent manner. Following incubation with compound **1** for 12 h, the morphology of B16 2F2 cells was markedly changed, and phospho-cofilin in the cells had completely disappeared. Activation of the p38 MAPK pathway by compound **1** results in the induction of melanogenesis in B16 2F2 cells. SB203580, a selective inhibitor of p38 MAPK, blocked the induction of tyrosinase in B16 2F2 cells by compound **1**; however, SB203580 did not influence the disruption of stress fiber assembly and the activation of cofilin in B16 2F2 cells stimulated with compound **1** (Fig. 9).

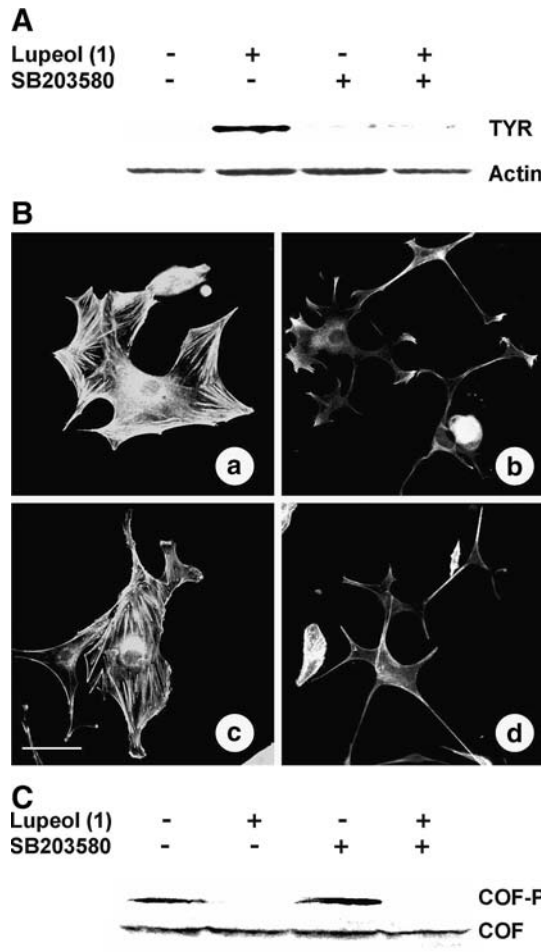


Fig. 9 Effects of a p38 MAPK inhibitor on markers of B16 2F2 cell differentiation. (**A**): B16 2F2 cells were cultured alone or with 10 μM compound **1**, 5 μM SB203580 (SB) or compound **1** + SB203580 for 48 h, and cell lysates were analyzed by Western blotting using anti-tyrosinase (TYR) antibody (upper panel) or anti-actin antibody (lower panel). (**B**): B16 2F2 cells were incubated alone (a) or with 10 μM compound **1** (b), 5 μM SB203580 (c) or compound **1** + SB203580 (d) for 12 h. The actin cytoskeleton was stained with fluorescent-labeled phalloidin (bar, 50 μm). (**C**): B16 2F2 cells were incubated alone, or with 10 μM compound **1**, 5 μM SB203580 (SB) or compound **1** + SB203580 for 12 h, and the cell lysates were analyzed by Western blotting using anti-phospho cofilin (COF-P) antibody (upper panel) or anti-cofilin (COF) antibody (lower panel)

Melanoma cell motility

It has been reported that disruptions in actin fiber assembly are involved in cancer cell motility and invasion (Byers et al. 1992). We investigated the effects of compound **1** on the cell motilities of several types of human cancer cells using Transwell cell culture chambers (Hata et al. 2005). The results are shown in Table 3. Compound **1** did not influence the growth of nine types of cancer cells, and weakly inhibited HeLa cell proliferation. Compound **1** markedly suppressed melanoma and neuroblastoma cell migration, which are derived from the neural crest, but did not inhibit the migration of other types of human cancer cells. Furthermore, it was revealed

Table 3 Effects of compound **1** on growth and migration of cancer cells

Cell line (origin)	Cell growth (%)	Migration index (%)
G361 (melanoma)	97.5 ± 3.8	40.5 ± 3.1
NB-1 (neuroblastoma)	96.0 ± 2.5	39.7 ± 6.1
A549 (lung adenocarcinoma)	100.1 ± 7.4	87.3 ± 5.0
ACHN (renal adenocarcinoma)	106.3 ± 6.9	103.4 ± 4.8
HeLa (cervical carcinoma)	72.4 ± 2.3	101.4 ± 4.1
HT1080 (fibrosarcoma)	91.6 ± 6.5	100.6 ± 10.8
MIA Paca2 (pancreatic cancer)	99.1 ± 4.9	93.1 ± 4.7
Saos2 (osteogenic sarcoma)	100.0 ± 9.8	101.3 ± 5.0
SH-10-TC (stomach cancer)	99.6 ± 5.2	94.6 ± 4.1
T24 (urinary bladder carcinoma)	90.7 ± 5.5	101.5 ± 2.3

The effects of 10 μM compound **1** on cell growth for 72 h and migration for 6 h were examined The values represent percentages, relative to the control ($n = 4$)

that compound **1** disrupted of stress fiber assembly in G361 melanoma cells, but did not influence the actin cytoskeleton of Saos2 osteogenic cancer cells, which are resistant to cell migration by compound **1**. Compound **1** might selectively disrupt the assembly of actin stress fibers in neural crest-derived cells, and this action results in its inhibitory effects on cell migration. Recently, it was shown that compound **1** exhibits an anti-angiogenic effect through the inhibition of HUVEC tube formation (You et al. 2003). In this study, we demonstrated the anti-migration activity of compound **1** against melanoma and neuroblastoma cells. These results suggest that compound **1** could be an effective compound to prevent tumor metastasis.

Conclusions

In the present study, it was revealed that lupane triterpenes induced melanogenesis and dendritic formations in mouse melanoma cells. Both melanogenesis and dendritic formation are known to be hallmarks of melanoma cell differentiation, but they were regulated separately by the activation of p38 MAPK signaling and inhibition of Rho signaling, respectively (Fig. 10).

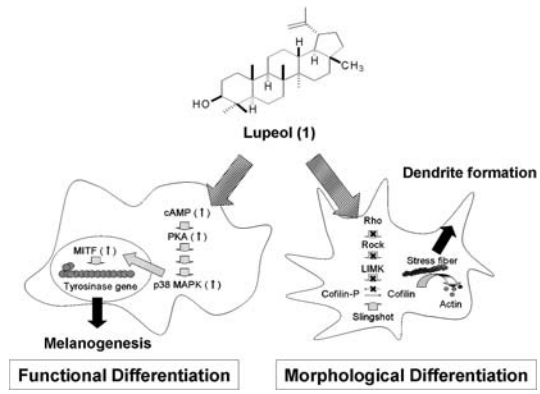


Fig. 10 Signaling mechanisms in compound **1**-induced B16 2F2 cell differentiation

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